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Identification of diphenhydramine metabolites in human urine by capillary electrophoresis-ion trap-mass spectrometry

The identification of diphenhydramine (DH) metabolites that are frequently observed in the capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC) analyses of alkaline liquid/liquid and solid-phase extracts of patient urines is demonstrated. Having standards for DH and diphenhydramine-*N*-oxide (DHNO), the presence of these two compounds could be confirmed in urines that were collected overnight after administration of 25 mg DH chloride. Using CZE coupled to ion-trap mass spectrometry (CE-MS^n) with positive electrospray ionization and an acetate buffer at pH 5.6, the $[M+H]^+$ ions of DH (*m/z* = 256), DHNO (*m/z* = 272), and nordiphenhydramine (NDH, *m/z* = 242) and their fragmentation to a common *m/z* 167 product ion (diphenylcarbinol moiety) was monitored. The data indicate that all three compounds are cations in an acidic environment, the migration order being NDH, DH, and DHNO. Data obtained under negative electrospray ionization conditions suggest the presence of diphenylmethoxyacetic acid-glycine amide ($[M-H]^-$ ion of *m/z* 298 and fragmentation to *m/z* 254, loss of CO_2), a metabolite that could tentatively be assigned to a characteristic peak observed in the MEKC electropherogram at alkaline pH. The data presented in this paper illustrate the value of using CE-MS^n for identification of urinary drug metabolites for which no standards are available.

Keywords: Desmethyldiphenhydramine / Diphenhydramine-*N*-oxide / Diphenylmethoxyacetic acid-glycine amide
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1 Introduction

Diphenhydramine (2-(diphenylmethoxy)-*N*,*N*-dimethylethylamine, DH, for chemical structure refer to Fig. 1) is an antihistaminic drug whose characteristics were first described in 1946. Beside the antihistaminic effect, DH is also used for its sedative and antiemetic properties and DH is administered orally as the hydrochloride salt [1]. A large number of preparations containing either DH alone or DH combined with another drug, including lorazepam and methaqualone here in Switzerland, are currently available. Thus, analysis of patient samples often reveals the presence of DH [2–6]. In our projects dealing with the capillary electrophoresis (CE) analysis of drugs of abuse in patient urines [3, 4] and methaqualone metabolites in urine after coadministration of methaqualone and DH [7–9], DH and two additional peaks with essentially the same

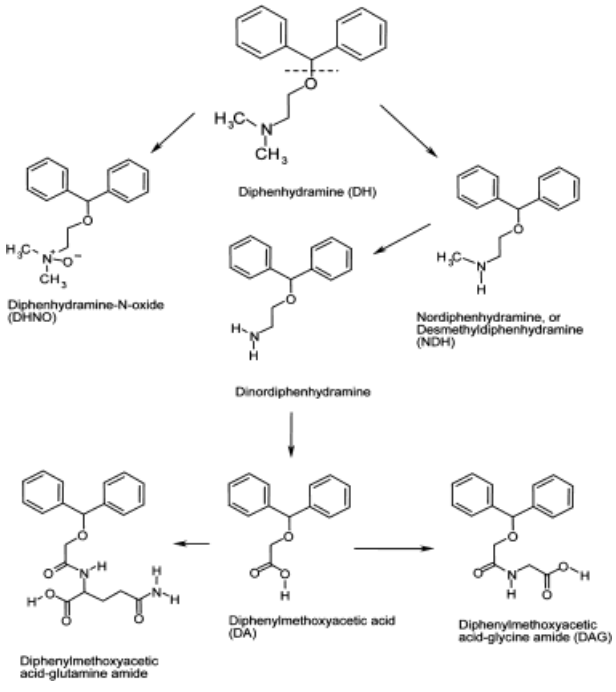

Figure 1. Chemical structures of DH and some of its expected metabolites. The dashed line in the chemical structure of DH marks the fragmentation to the diphenylcarbinol moiety.

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Abbreviations: DA, diphenylmethoxyacetic acid; **DAG**, diphenylmethoxyacetic acid-glycine amide; **DH**, diphenhydramine; **DHNO**, diphenhydramine-*N*-oxide; **NDH**, nordiphenhydramine

UV spectra as DH were typically monitored. These peaks are seen in the micellar electrokinetic capillary chromatography (MEKC) data presented in Fig. 2, data that were obtained for the analysis of an extract of a patient urine who was under a DH and lorazepam combination pharmacotherapy (Somnium; Medichemie, Ettingen, Switzerland). Two additional peaks with the same absorption spectra as DH were also seen by high-performance liquid chromatography (HPLC) and were thus assumed to be those of DH metabolites. Due to the lack of standards, however, the metabolites could not be identified.

The metabolism of DH has been investigated quite some time ago [1, 2, 10, 11]. DH undergoes an extensive oxidative metabolism *via N*-demethylation to the secondary (nordiphenhydramine, desmethyldiphenhydramine, NDH) and subsequently to the primary (dinordiphenhydramine) amine, both excreted in the urine. Dinordiphenhydramine is further oxidized to diphenylmethoxyacetic acid (DA) which appears to become conjugated with glycine (formation of diphenylmethoxyacetic acid-glycine amide (DAG)) or glutamine prior to excretion. Another reported metabolic step of DH is the biotransformation to diphenhydramine-*N*-oxide (DHNO; Fig. 1). The half-life of DH in plasma was found to be age-dependent, with values of

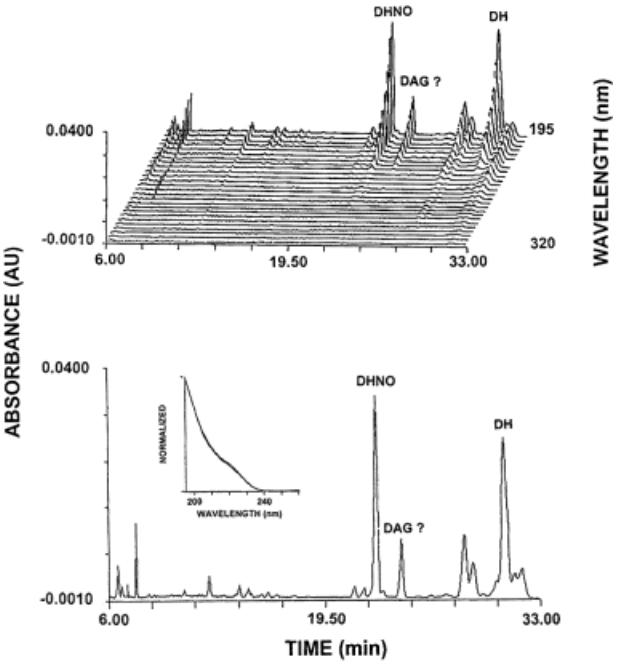

Figure 2. MEKC (A) multiwavelength electropherograms between 195 and 320 nm (5 nm interval) and (B) single-wavelength electropherogram at 200 nm obtained for an alkaline SPE extract of a patient urine who was under pharmacotherapy with Somnium (data taken from Fig. 5B of [3]). The inset in panel B depicts normalized spectra of DH and the two metabolites. For experimental conditions refer to [3].

5.4, 9.2 and 13.5 h for children, adults and elderly persons, respectively. After 96 h, about 64% of the administered dose is eliminated as urinary metabolites [10]. Over the years, various methods have been used to monitor DH and selected metabolites in body fluids, including thin-layer chromatography and gas chromatography-mass spectrometry [11], HPLC with UV-absorbance detection [2,5,6], liquid chromatography-mass spectrometry (LC-MS) [12], MEKC with multiwavelength UV-absorbance detection [4] and capillary zone electrophoresis (CZE) with absorbance [7] or electrochemiluminescence [13] detection. Little effort has been focused on the determination of DH metabolites in urine [11].

The goal of this project was to identify the DH metabolite peaks in our MEKC [3, 4] and CZE [7–9] electropherograms using CZE coupled to ion-trap MS (CE-MS^n), a technology that was recently employed for the identification of urinary oxycodone metabolites [14]. Except for DHNO that was synthesized in-house, metabolite standards were not available. Nevertheless, the combination of data obtained by CZE, MEKC and CE-MS^n is shown to allow the identification of the metabolites leading to the characteristic peaks in the electropherograms obtained with alkaline extracts of urines that were collected after administration of DH.

2 Materials and methods

2.1 Chemicals and urines

DH (as hydrochloride salt) and KH_2PO_4 were from the Inselspital Apotheke (Bern, Switzerland). 3-Chloroperbenzoic acid, sodium dodecyl sulfate (SDS) and H_3PO_4 85% were from Fluka (Buchs, Switzerland) and $Na_2HPO_4 \times 2\ H_2O$, $Na_2B_4O_7 \times 10\ H_2O$, ammonium acetate, ammonia, acetic acid, formic acid, *n*-hexane, isopropanol, and chloroform were all of 'per analysis' grade and were from Merck (Darmstadt, Germany). Methanol and dichloromethane (both HPLC grade) were purchased from Biosolve (Valkenswaard, The Netherlands), aluminum oxide for chromatography (No. 015.0811) was from Camag (Muttenz, Switzerland) and bidistilled water was obtained from a Fitstream™ Cyclon™ apparatus (Loughborough, Great Britain). Urine samples analyzed stemmed from two individuals who gave their consent and took one tablet of Toquilone compositum (Medichemie, Ettingen, Switzerland) containing 25 mg DH chloride and 250 mg methaqualone and whose urines were collected overnight (WT0-8 h and SZ0-9 h, respectively). These urines have been analyzed before for methaqualone and its metabolites by CZE in acidic buffers containing hydroxypropyl-β-cyclodextrin [7–9].

2.2 Urine preparation

Two extraction procedures were employed. DH and its metabolites were extracted at pH 9 using liquid/liquid extraction with Toxi-Tubes A (Varian, Laguna Hills, CA, USA). Urine (2.5 mL) was diluted with 2.5 mL bidistilled water and placed into the Toxi-Tube. The tube was then manually shaken for 30 s and centrifuged at 1500 × *g* for 3 min. An aliquot of 1.7 mL of the organic layer was transferred into a clean glass tube and evaporated to dryness at 37°C under a gentle stream of nitrogen. The sample was reconstituted in 100 μL water. Furthermore, a previously described [4,8,9,15] two-step solid-phase extraction (SPE) scheme using mixed mode Bond Elut Certify (No. 1211-3050; Varian, Harbor City, CA, USA) disposable cartridges together with a Vac-Elut setup (Varian) was employed. All the required buffers and solutions were prepared according to the instructions contained in the instruction manual. Briefly, a cartridge was conditioned with 2 mL methanol and 2 mL phosphate buffer at pH 6. Then, the sample, consisting of 2.5 mL urine, 2.5 mL water and 2 mL phosphate buffer at pH 6 was loaded and slowly drawn through the cartridge. The cartridge was sequentially rinsed with 1 mL phosphate buffer, pH 6/methanol (80:20 v/v), 1 mL 1 M acetic acid, 1 mL *n*-hexane. Analyte withdrawal occurred first with 4 mL methylene chloride (first fraction, discarded), then with 6 mL methanol, and finally with 2 mL of a solution composed of methanol/concentrated ammonia (70:30 v/v) (second fraction). The second eluate was evaporated to dryness at 37°C under a gentle stream of nitrogen. The sample was reconstituted with 100 μL of water (for CE-MS^n and MEKC analysis) or 10-fold diluted running buffer (for CZE analysis) and stored at −20°C.

2.3 CE-MS^n instrumentation and running conditions

MS was performed on a Finnigan LCQ ion-trap instrument (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray ionization interface (Finnigan). Experiments were performed in the positive and negative ionization modes. The sheath gas (N_2) pressure was set at 23 or 20 arbitrary units for positive and negative analyses, respectively. The sheath liquid consisted of a mixture of water/methanol in proportion 1:1 (v/v) containing 1.1% of formic acid (or 0.4% concentrated ammonia) in order to support the formation of positively (negatively) charged ions. It was infused at a flow rate of 5.0 μL/min using a 250 μL Unimetrics syringe. The applied voltages were 4 kV (positive mode) and −3.5 kV (negative mode). The temperature of the heated capillary was kept at 200°C. The instrument was computer-controlled using the Xcalibur 1.2 software (Finnigan). A Prince Instrument (Lauerlabs, Emmen, The Netherlands) equipped with a 75 μm ID fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 80 cm length was interfaced to the LCQ. The sample was introduced hydrodynamically by applying a positive pressure (70 mbar, 30 s). The background electrolyte was prepared daily as follows. Ammonium acetate powder was dissolved in water in order to obtain a concentration of 20 mM followed by the adjustment of the pH to 5.6 with 1 M acetic acid. The applied voltage during separation was 30 kV. Full scan mass spectra were collected in the mass range of 100–600 Thompson (Th). Automatic gain control (AGC) was employed using three microscans and a maximum injection time of 200 ms. MS^2 experiments were performed targeting selectively the *m/z* ratios of the compounds of interest (Table 1) with an isolation width of 2 Th, and applying a collision energy of 30 or 35.

Table 1. Masses and CE-MS identification of investigated urinary compounds

Compound	Molecular mass[a)]	CE-MS with positive ionization			CE-MS with negative ionization		
		$[M+H]^+$ ion[a)]	SPE[b)]	l/l[b)]	$[M-H]^-$ ion[a)]	SPE[b)]	l/l[b)]
DH	255.36	256.37	D,C	D,C	254.35	ND	ND
NDH	241.34	242.35	D,C	D,C	240.33	ND	ND
Dinordiphenhydramine	227.31	228.32	ND	ND	226.30	ND	ND
DHNO	271.36	272.37	D,C	D,C	270.35	ND	ND
DA	242.28	243.29	ND	ND	241.27	ND	ND
DAG	299.33	300.34	Trace	ND	298.32	D,C?	Trace
Diphenylmethoxyacetic acid-glutamine amide	370.41	371.42	ND	ND	369.40	ND	ND

a) Calculated using the ISIS DRAW Version 2.1.3d software (MDL Information Systems, San Leandro, CA, USA), for chemical structures see Fig. 1.

b) D, ND and C refer to detected, not detected and confirmed after SPE or liquid/liquid (l/l) extraction, respectively, of urine WT0-8h. Confirmation was based upon CE-MS^2. Trace refers to the detection of a very weak signal of the expected mass that could not be confirmed by CE-MS^2.

2.4 MEKC and CZE conditions

MEKC and CZE experiments were performed on a Bio Focus 3000 instrument (Bio-Rad Laboratories, Hercules, CA, USA) that was equipped with a 50 μm ID fused-silica capillary of 45.5 cm effective length (50 cm total length). The capillary and the carousels were kept at 25°C. The MEKC buffer was the same as used previously [3, 4] and consisted of 6 mM $Na_2B_4O_7 \times 10\ H_2O$, 10 mM $Na_2HPO_4 \times 2H_2O$, 75 mM SDS, and isopropanol (aqueous/organic solvent proportion of 95/5 v/v). The pH measured prior to the addition of SDS and isopropanol was 9.2. The sample was injected hydrodynamically at 10 psi·s. CZE experiments were performed with a 75 mM KH_2PO_4 buffer whose pH was adjusted to 2.5 with H_3PO_4 and a 20 mM ammonium acetate buffer whose pH was adjusted to 4.6 with acetic acid. The samples were injected for 4 psi·s. For solute identification purposes, the fast scanning detection mode (range: 195–320 nm at 5 nm intervals) was employed as previously described [3, 4].

2.5 Synthesis of DHNO

The synthesis of DHNO was performed according to the protocol employed by Craig and Purushothaman [16] for the synthesis of other tertiary amine *N*-oxides. The proposed procedure was followed except for the elimination of the unreacted parent compound. 0.1469 g (*ca.* 0.5 mmol) of DH hydrochloride was placed in a 5 mL disposable glass tube, dissolved in 2.0 mL chloroform and the tube was placed on ice. 0.0914 g of 3-chloroperbenzoic acid (*ca.* 0.5 mmol) was dissolved in 1 mL chloroform and slowly added to the cold DH solution while stirring. About 10 min after completion of the mixing, the tube was allowed to reach room temperature and the mixture was periodically stirred during the next 3 h. Thereafter, the solution was purified using a hand-packed column of aluminum oxide (*ca.* 20 times the weight of the combined reagents put into a plastic syringe) conditioned with 10.0 mL of chloroform. The reaction mixture was slowly loaded, the column was rinsed with 10 mL chloroform and the elution was performed with 10 mL methanol/chloroform (1/3 v/v). Finally, the eluate was evaporated to dryness at 40°C under a gentle stream of air, reconstituted with 300 μL of methanol and stored at −20°C. The identities of DHNO and DH were verified by MS^n (up to n = 3) in the positive ionization mode and using the Prince for sample injection without application of an electric field (for MS conditions see Section 2.3). The observed mass/charge ratios were 272 and 256, corresponding to the theoretical $[M+H]^+$ *m/z* ratios of DHNO and DH, respectively (Table 1). Both mass spectra also exhibited peaks at *m/z* = 167, the fragment ion that is produced after isolation and fragmentation of the $[M+H]^+$ ions (Fig. 3). This common *m/z* 167 fragment represents the diphenylcarbinol moiety as indicated with the dashed line in the chemical structure of DH (Fig. 1). The precursor-product ion transitions for DH (256 → 167) and DHNO (272 → 167) are the same as previously reported using LC-MS [4]. The MS^2 mass spectrum of DHNO also shows a fragment with *m/z* = 183, probably due to fission of the C-O bond and loss of the side chain (Fig. 3D).

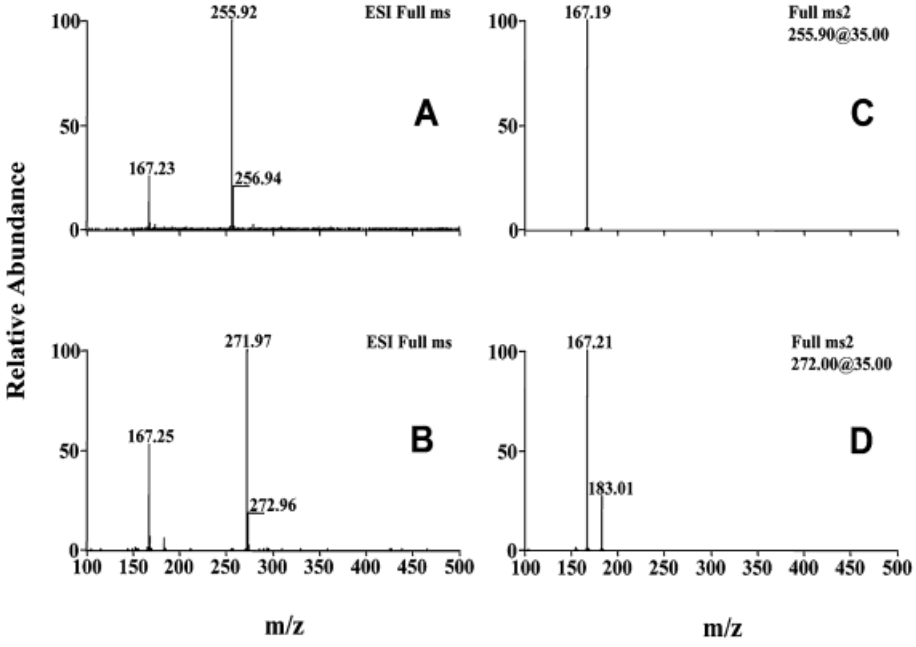

Figure 3. (A), (B) MS data and (C), (D) MS^2 data of (A), (C) DH and (B), (D) DHNO obtained *via* infusion of the sample into the electrospray ionization interface using the Prince instrument.

3 Results and discussion

3.1 Analysis of DHNO by MEKC and CZE with UV-absorbance detection

With the availability of standards for DH and DHNO, these two compounds could easily be detected by MEKC at alkaline pH (Fig. 4A) and CZE at low pH (Fig. 4B). Peaks were identified by spiking the extract with the standard compounds and reanalysis. The first metabolite peak in the MEKC electropherogram could thereby be identified as DHNO. In CZE at pH 2.5 (Fig. 4B), DHNO was detected shortly after DH, indicating that DHNO is positively charged at that pH. The normalized spectra of the two compounds were found to be identical (for an example refer to inset in Fig. 2B). Using the ammonium acetate buffer at pH 4.6, DH was detected as cation well before the electroosmotic peak, whereas DHNO was found to migrate shortly before the void peak suggesting that the *N*-oxide group is partially protonated at pH 4.6 (data not shown).

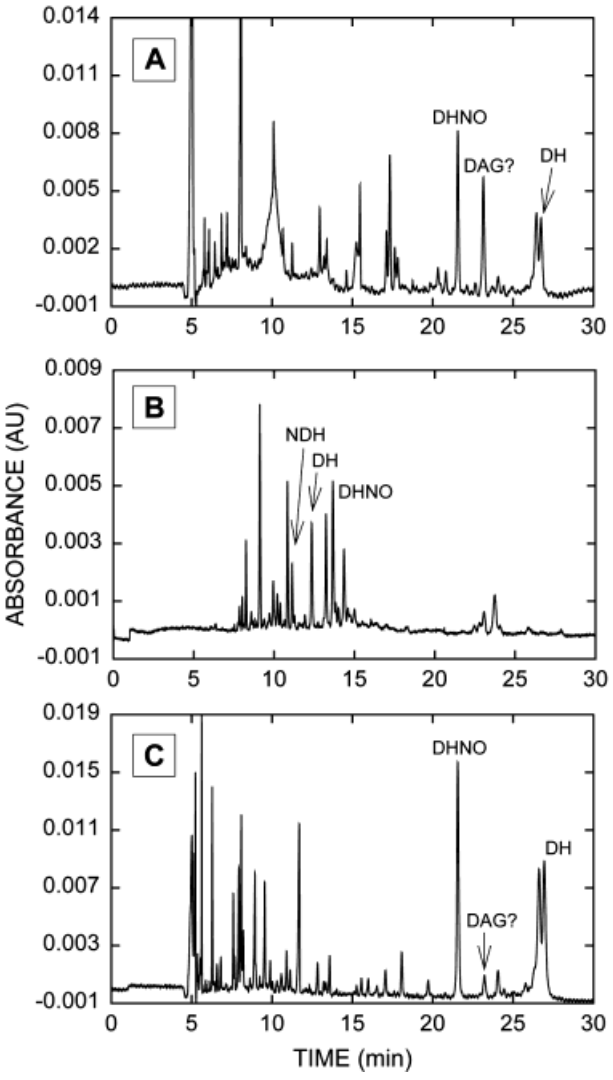

Figure 4. Electropherograms obtained at 200 nm for urine WT0–8h with (A) MEKC (SPE with sample reconstituted in H_2O); (B) CZE at pH 2.5 (SPE with sample reconstituted in 10-fold diluted phosphate running buffer); and (C) MEKC (liquid/liquid extraction with sample reconstituted in water). Applied voltages were (A), (C) 20 kV and (B) 14 kV, and currents were (A), (C) 37 μA and (B) 46 μA. For experimental conditions refer to Section 2.4.

Spectra comparable to those of DH and DHNO were also observed for the second metabolite peak in the MEKC electropherograms (inset in Fig. 2B; Fig. 4A) and for the CZE peak detected at about 11.1 min in the data presented in Fig. 4B. Thus, it was assumed that these peaks are DH metabolites as well. The second metabolite of the MEKC data was found to become much better extracted by SPE with the mixed-mode phase compared to liquid/liquid extraction (compare data of Figs. 4A and C). This suggests the presence of a metabolite whose chemical properties are different from DH and NDH, such as DA or DAG (Fig. 1). Furthermore, NDH is extracted well using both procedures. NDH is a slightly smaller molecule than DH and is thus expected to be detected before DH in a CZE-based electropherogram. The small peak in Fig. 4B monitored at about 11.1 min could be an indication of the presence of this metabolite. Thus, liquid-liquid and solid-phase extracts were analyzed by CE-MS^n with the purpose of determining the nonassigned peaks in the electropherograms.

3.2 CE-MS^n with positive electrospray ionization

The compounds looked for by CE-MS^n were those whose chemical structures and *m/z* values are presented in Fig. 1 and Table 1, respectively. In the analysis of the SPE extract of WT0–8h urine in the positive ionization mode (Fig. 5), mass traces for *m/z* values of 256, 242 and 272 corresponding to the masses of the $[M+H]^+$ ions of DH, NDH and DHNO, respectively, were found (Figs. 5B, C, D, respectively). The mass traces indicate that NDH is migrating at the front edge of DH (panels B, C). This was confirmed with the mass spectra at these time points that were found to contain the masses for both compounds and a common fragment ion (see below, for mass spectra refer to panels E and F of Fig. 5). DHNO is detected much later at 8.48 min (panel G). Collision-induced dissociation of each of these ions led to precursor-product ion transitions between 256 → 167, 242 → 167, 272 → 167, respectively, (for examples see Fig. 3) which confirm the presence of DH, NDH and DHNO, respectively, in the urine. The common *m/z* 167 fragment (the diphenylcarbinol moiety) is already present in the mass spectra of

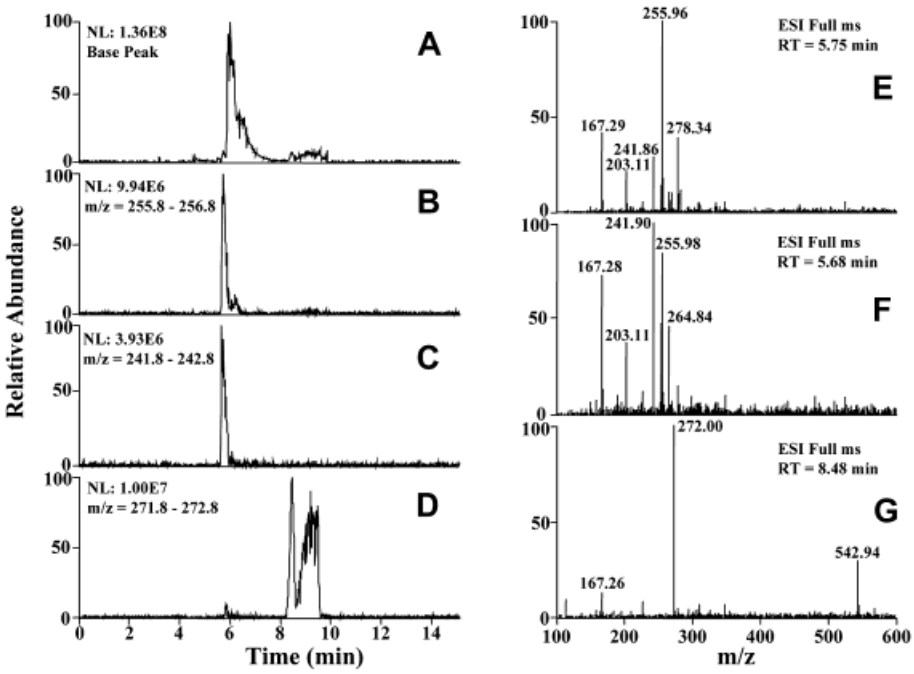

Figure 5. CE-MS data with positive electrospray ionization obtained with urine WT0-8h after SPE and sample reconstitution in water. (A)–(D) depict the base peak and mass traces for *m/z* of 256, 242, and 272, respectively, whereas (E)–(G) depict MS spectra at 5.75, 5.68 and 8.48 min of the data of (B)–[D), respectively. The three mass spectra indicate the presence of DH, NDH and DHNO, respectively. For experimental conditions refer to Section 2.3.

Figs. 5E–G. Furthermore, a trace of a mass/charge ratio corresponding to that of DAG (*m/z* = 300) was found at 9.6 min (data not shown), but the signal was too close to the baseline noise to either perform a fragmentation or to make any reliable assumption about this signal. Comparable findings were obtained with liquid/liquid extracts. Dinordiphenhydramine, DA and diphenylmethoxyacetic acid-glutamine amide could not be detected in the two extracts (Table 1).

3.3 CE-MS^n with negative electrospray ionization

In the case of positive ionization, the MS was tuned with DH. As DH could not be detected with negative ionization, ibuprofen was employed as tuning compound in that mode. Assuming the presence of DAG in the extracts, it appeared meaningful that negative ionization would result in a more selective determination of this compound. Furthermore, the fragmentation reaction was expected to follow a different pattern than that observed in the positive ionization mode. The most probable site of deprotonation would be located on the carboxylic group of the glycine residue of DAG, and a likely fragmentation would feature a loss of a neutral CO_2 molecule, with the resulting fragment being stabilized by resonance (Fig. 6). Analysis of both extracts revealed a peak corresponding to the $[M\text{-}H]^-$ ion of DAG (*m/z* = 298) (data obtained after SPE are depicted in Figs. 7B and C), the peak in the liquid/liquid extract, however, being much smaller than the one in the SPE extract. No other mass of interest was detected in this mode (Table 1).

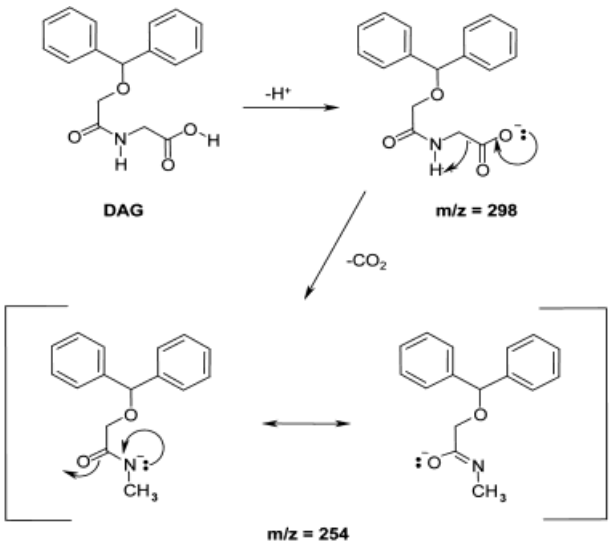

Figure 6. Proposed mechanism for the fragmentation of DAG after negative electrospray ionization.

The signal obtained for the SPE extract was strong enough to be fragmented and thereby analyzed in the MS^2 format. Fragmentation of the $[M\text{-}H]^-$ ion was found to provide two product ions with *m/z* values of 254 and 176 (Fig. 7D). MS^3 of the 254 ion led to a single fragment

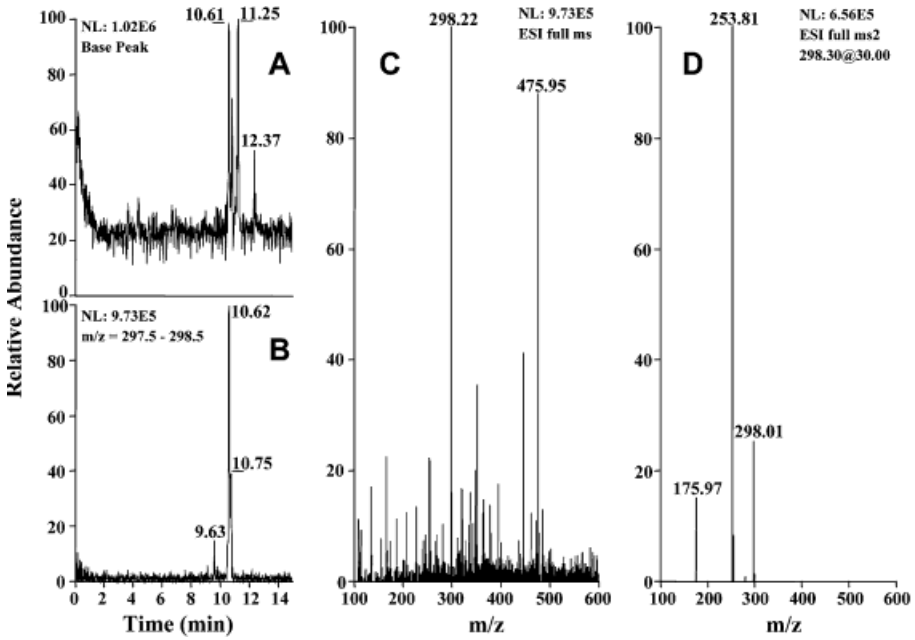

Figure 7. CE-MS data with negative electrospray ionization obtained with urine WT0-8h after SPE and sample reconstitution in water. (A), (B) depict the base peak and mass trace for *m/z* of 298, respectively, (C) depicts the MS spectrum at 10.62 min of the data of (B) and (D) shows MS^2 data obtained after isolation of the 298 ion of (C). For experimental conditions refer to Section 2.3.

with *m/z* = 176 (data not shown). The 298 → 254 transition is in agreement with the proposed mechanism, *i.e.*, the loss of CO_2 (Fig. 6). The stronger presence of this compound in the SPE extract compared to that prepared by liquid/liquid extraction, a fact that was observed for one peak in the MEKC electropherograms as well (compare data of Figs. 4A and C), suggests that the second metabolite peak appearing in the MEKC electropherogram between DHNO and DH (Figs. 2, 4A and C) could be DAG. As the MEKC buffer is unsuitable for $CE\text{-}MS^n$ and we were not in a position to collect a fraction with the peak in question for subsequent MS analysis, however, a complete proof cannot be obtained without the availability of a DAG standard. NDH was found to become extracted equally in the two extraction procedures and can thus be excluded for assignment of the second metabolite peak in the MEKC electropherogram.

4 Synopsis and concluding remarks

Due to the availability of standards for two of the compounds, namely DH and DHNO, these compounds could be unambiguously identified after liquid-liquid and solid-phase extraction of urine that was collected overnight after administration of 25 mg DH. Using CE-MS, the presence of these two compounds could be confirmed. No standards were available for the other metabolites such that they had to be analyzed with $CE\text{-}MS^n$ and assigned *via* analogy considerations from information gained under different extraction and CE configurations. The $CE\text{-}MS^n$

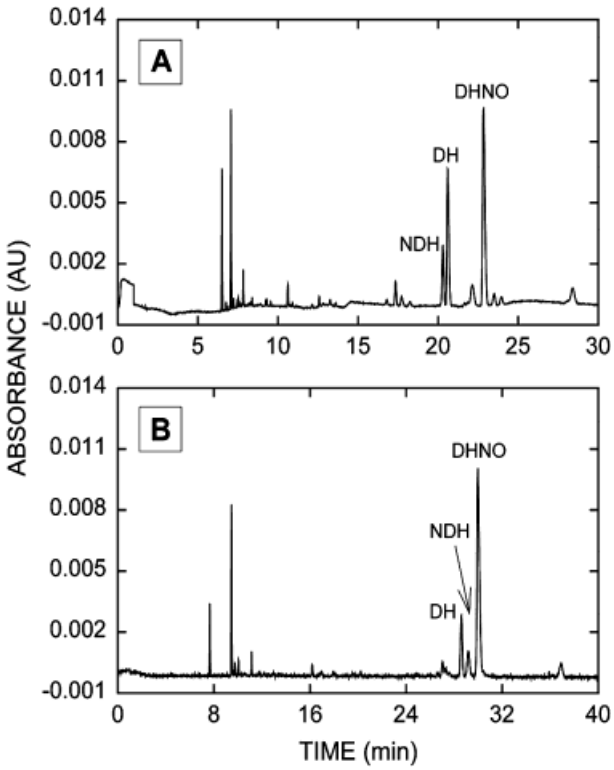

Figure 8. Electropherograms obtained with liquid/liquid extracts of urine WT0-8h using (A) a pH 2.5 phosphate buffer containing 8 mM hydroxypropyl-β-cyclodextrin (adapted from Fig. 5B of [7]) and (B) a pH 2.1 phosphate buffer with 50 mM hydroxypropyl-β-cyclodextrin (adapted from Fig. 7A of [8]). For experimental conditions refer to [7] and [8], respectively.

data revealed that NDH was extracted and detected. It's migrational behavior at pH 5.6 was noted to be very similar to that of DH. As NDH is somewhat smaller than DH and otherwise of very similar chemical structure it did not come as a surprise that NDH was detected at the front edge of DH. It is meaningful to assume that NDH is migrating ahead of DH at pH 2.5 and we are thus inclined to assign the peak at about 11.1 min in Fig. 4B to NDH. The same should apply for the data presented in [7] in which a similar urinary extract was analyzed in a pH 2.5 phosphate buffer containing 8 mM hydroxypropyl-β-cyclodextrin (Fig. 8A). Interestingly, having 50 mM hydroxypropyl-β-cyclodextrin and a buffer pH of 2.1 [8], the peak assigned to NDH was detected between DH and DHNO (Fig. 8B). DAG, with its acidic pK_a value assumed to be very similar to that of glycine (pK_a = 2.4), is negatively charged at low pH and cannot be detected by CZE with the buffers employed here. In MEKC at alkaline pH, the second metabolite peak could tentatively be assigned to DAG and NDH is assumed to coelute with DH. The data presented in this paper illustrate the suitability of CE-MS^n for identification of urinary drug metabolites for which no standards are available.

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